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Partial reconstitution of DNA large loop repair with purified proteins from *Saccharomyces cerevisiae*

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ABSTRACT

Small looped mispairs are corrected by DNA mismatch repair. In addition, a distinct process called large loop repair (LLR) corrects heteroduplexes up to several hundred nucleotides in bacteria, yeast and human cells, and in cell-free extracts. Only some LLR protein components are known, however. Previous studies with neutralizing antibodies suggested a role for yeast DNA polymerase δ (Pol δ), RFC and PCNA in LLR repair synthesis. In the current study, biochemical fractionation studies identified FEN1 (Rad27) as another required LLR component. In the presence of purified FEN1, Pol δ , RFC and PCNA, repair occurred on heteroduplexes with loops ranging from 8 to 216 nt. Repair utilized a 5' nick, with correction directed to the nicked strand, irrespective of which strand contained the loop. In contrast, repair of a G/T mismatch occurred at low levels, suggesting specificity of the reconstituted system for looped mispairs. The presence of RPA enhanced reactivity on some looped substrates, but RPA was not required for activity. Although additional LLR factors remain to be identified, the excision and resynthesis steps of LLR from a 5' nick can be reconstituted in a purified system with FEN1 and Pol δ , together with PCNA and its loader RFC.

INTRODUCTION

Prokaryotic and eukaryotic cells are capable of repairing DNA loop mismatches ranging from one to over 5000 nt in length (1–8). Two major pathways are involved in the correction of these heterologies: mismatch repair (MMR) and large loop repair (LLR). Although there appears to be some substrate overlap between the two, MMR corrects loops of 1 to ~8–17 nt, depending on the species (9–14). The bacterial MMR system consisting of the MutS, MutL, MutH and UvrD proteins can efficiently repair loops up to

about 4 nt (15). Although heterologies from 5 to 8 nt are also repaired, the efficiency decreases as the loop size increases (11). Eukaryotic MMR uses the MutS homolog (MSHs) and the MutL homologs (MLHs). In yeast and humans, MMR can repair loops up to ~16–17 nt (13,14). As in bacteria, repair efficiency decreases with increasing loop size.

The poorly defined LLR pathway corrects loops larger than those repaired by MMR and has been reported in *Escherichia coli*, yeast and mammalian cells. *In vivo* evidence for LLR in *E. coli* includes transfection experiments showing that MMR-deficient bacteria can repair a heteroduplex containing a 800-nt loop (2). *In vitro* experiments using extracts deficient in the MutH, MutL and MutS proteins demonstrated correction of loops up to 429 nt in length (16). Transformation, microinjection and gene targeting experiments using mouse and monkey cells revealed that mammalian cells can repair loops as large as 2.8 kb (3,4,17,18). *In vitro* studies using extracts from human cells have indicated that loops as large as 993 nt can be repaired (12,13,19,20). Extracts deficient in the mismatch repair proteins Msh2, Msh6, Mlh1, Pms2, the nucleotide excision repair proteins XPA, XPG, XPF/ERCC4 and ERCC1, and in Werner syndrome protein (WRN) are proficient at rectifying large loops, which suggests they may be dispensable for LLR (12,13,19). To date, none of the proteins involved in the repair of large loops in *E. coli* or mammalian cells has been identified.

The capacity of the yeast *Saccharomyces cerevisiae* to repair large loops is well documented (6,8–10,14,21–26). However, LLR in yeast appears to be a complex process, as three distinct LLR pathways have been described. Two of these pathways appear to function only during meiotic LLR. One meiotic LLR pathway that can repair heterologies as large as 5.6 kb employs the MMR proteins Msh2 and Msh3 and the NER endonucleases Rad1 and Rad10 (6,8). A second meiotic pathway has been postulated since meiotic LLR still occurs in strains in which *RAD1* is deleted (6,8). The third LLR pathway functions in proliferating cells, and it has been demonstrated *in vivo* and in nuclear extracts (23,25). Although these studies did not

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identify the proteins involved in mitotic LLR, the use of deletion mutants suggested that Msh2, Msh3, Mlh1, Pms1, Rad1, Rad2 and Rad27 may be dispensable (21,23,25).

Based on neutralizing antibody experiments with yeast extracts, PCNA, DNA polymerase δ (Pol δ) and replication factor C (RFC) were implicated in DNA repair synthesis necessary for LLR (27). To identify additional LLR components, we fractionated extracts to look for proteins that reconstituted LLR when supplemented with purified PCNA, Pol δ and RFC. Mass spectrometric analysis of such a fraction revealed the presence of flap endonuclease 1 (FEN1; Rad27p). Using purified proteins, we present evidence that FEN1, Pol δ , PCNA and RFC are sufficient for 5' nick-directed repair of heteroduplex substrates containing 8–216 nt loops. To our knowledge, this is the first report of a biochemically defined system that can support LLR *in vitro*.

MATERIALS AND METHODS

Construction of DNA substrates

The substrates are summarized in Supplementary Table 1. Heteroduplexes containing a G/T mismatch and unstructured loops of 8, 16, 27, 30, 45 and 216 nt were prepared from ϕ 1 bacteriophages as previously described (23,25,28). Each substrate contained a site-specific nick generated by cleavage with *Sau96I* 114 bp 5' to the loop. We use the following nomenclature to describe the substrates: C substrates contain a loop on the complementary strand of the heteroduplex, while V substrates contain a loop on the viral strand. The numeric descriptor indicates the number of unpaired bases. For example, C₂₇ refers to a substrate with a 27-nt loop on the outer complementary strand, while V₂₇ contains a 27-nt loop on the inner viral strand (Figure 1).

Yeast strains

Nuclear extracts were prepared from strain DY6 (*MATa ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407*) obtained from B. Jones (Carnegie-Mellon University) via T. Hsieh (Duke University). Strain BY4741 (*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0*) and its isogenic derivative Δ rad27 were from Open Biosystems, Huntsville, Alabama, U.S.A. W303-1a (*MATa leu2-3,112 trp-1 ura3-1 can1-100 ade2-1 his3-11, 13*) and its isogenic derivative Δ rad2 Δ rad27 Δ yen1 Δ din7 were kindly provided by L. Symington (Columbia University).

Nuclear extract preparation

Nuclear extracts were prepared by a modification of the method of Wang *et al.* (29) as described (23). Briefly, 151 of cells were grown to A₆₀₀ of approximately 2.0, harvested, washed and incubated with zymolyase to produce spheroplasts. The spheroplasts were lysed with a homogenizer and intact nuclei were isolated by differential centrifugation through a Ficoll gradient. Nuclear proteins were extracted by the addition of NaCl to a final concentration of 0.2 M. After precipitation with ammonium sulfate, the proteins were dialyzed against 20 mM HEPES-KOH,

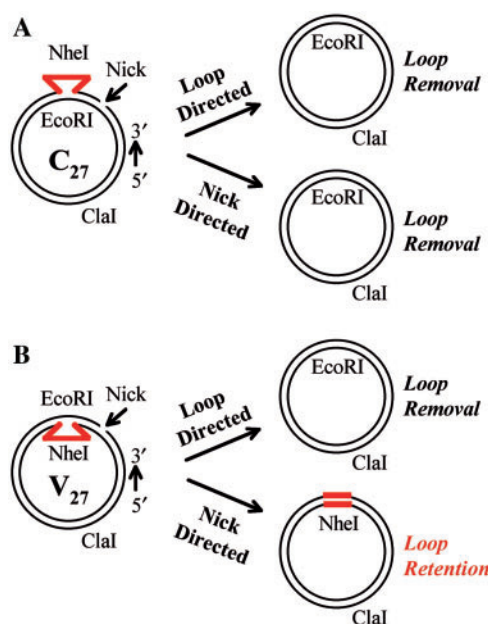


Figure 1. C₂₇ and V₂₇ heteroduplex substrates used for *in vitro* large loop repair assays. The C₂₇ substrate (A) contains a 27-nt loop and a 5' nick on the complementary (C) strand. An NheI site is on the strand containing the loop, while an EcoRI site is on the opposite strand. The presence of the loop renders the DNA resistant to digestion by either NheI or EcoRI. Therefore, double digests of ClaI with either NheI or EcoRI result in a full-length (6.4 kb) linear product. If repair of C₂₇ is targeted by the loop (top arrow) or by the nick (bottom arrow), both become sensitive to EcoRI. The V₂₇ substrate (B) contains a 27-nt loop on the viral (V) strand and a nick on the complementary strand. Loop-directed repair of V₂₇ results in loop removal and sensitivity to EcoRI. Nick-directed repair results in retention of the loop and sensitivity to NheI.

pH 7.6, 10 mM MgSO₄, 10 mM EGTA, 5 mM DTT, 20% glycerol, 2 μ M pepstatin A, 0.6 μ M leupeptin, 2 μ g/ml chymostatin, 2 mM benzamidine hydrochloride and 1 mM PMSF.

Whole-cell extract preparation

Whole-cell yeast extracts were prepared using a modification of the method described by Schultz *et al.* (30). Cells were harvested, weighed and successively washed with ice-cold distilled water and wash buffer containing 100 mM HEPES-KOH, pH 7.6, 5 mM EGTA, 1 mM EDTA, 20 mM KCl, 2.5 mM DTT and the protease inhibitors 2 μ M pepstatin A, 0.6 μ M leupeptin, 2 μ g/ml chymostatin, 2 mM benzamidine hydrochloride, 1 mM PMSF and 10 mM NaHSO₃. After centrifugation, the cell pellet was resuspended in 1.3 vol of extraction buffer (100 mM Tris-OAc, pH 7.9 containing 50 mM KOAc, 10 mM MgSO₄, 2 mM EDTA, 20% glycerol, 2.5 mM DTT and protease inhibitors), loaded into a syringe and extruded into liquid nitrogen. The frozen cells were broken by grinding with a mortar and pestle under liquid nitrogen. The cell powder was thawed into 0.5 vol of 20 mM HEPES-KOH, pH 7.6 containing 10 mM MgSO₄, 10 mM EGTA, 20% glycerol, 5 mM DTT and protease inhibitors. The lysate was centrifuged 20 min at 25 000g. The supernatant was assayed for LLR activity.

Loop repair assays

The repair of DNA loops by nuclear extracts and whole-cell lysates was monitored as described (12,23,25). Unless specified otherwise, repair reactions monitoring loop removal contained 20 mM HEPES-KOH, pH 7.6, 1 mM glutathione, 1.5 mM ATP, 0.1 mM (each) dATP, dGTP, dTTP and dCTP, 0.05 mg/ml BSA, 100 ng (24 fmol) of heteroduplex DNA and 40 µg nuclear extract or whole-cell lysate in a final volume of 10 µl. Loop retention assays contained 200 ng (48 fmol) of substrate and 80 µg of nuclear extract of cell lysate in a final volume of 20 µl. Following incubation at 30°C for 60 min, the reactions were quenched by the addition of 30 µl of 25 mM EDTA. The DNA was purified by phenol extraction and ethanol precipitation before incubation with the appropriate restriction enzymes. All restriction digests contained 6 U ClaI to linearize the DNA. Six units of XhoI was used to evaluate repair that favored loop retention of the V₈, C₈, V₃₀, C₃₀, V₄₅ and C₄₅ substrates. Five units of PvuII was used to score for V₃₀ and C₃₀ loop removal. Reactions scoring for V₄₅ and C₄₅ loop removal contained 2 U HindIII. Three units of XcmI was used in C₈ removal reactions. Five units of EcoRI was used in reactions monitoring V₂₇, C₂₇, V₁₆ and V₂₁₆ loop removal. V₂₇, C₂₇, V₁₆ and V₂₁₆ loop retention assays employed 2.5 U of NheI. Repair of the G/T mismatch to the A/T repair product was monitored with HindIII as described by (31). Following restriction digestion at 30°C for 1 h, repair products were separated on a 1% agarose gel, stained with ethidium bromide and quantitated with a Kodak EDAS 290 system. Bands were displayed as the photographic negative for ease of viewing. Repair activity is defined as the amount of product (3.3 + 3.1 kb bands) divided by the total DNA (6.4 + 3.3 + 3.1 bands) × 100%.

For assays using purified proteins, the phenol extraction and ethanol precipitation steps were omitted. Following incubation at 30°C, the reactions were terminated by transferring the mixtures to Eppendorf tubes preheated to 70°C, where they were incubated for 10 min. The mixtures were cooled on ice and 1 µl 10 × restriction enzyme buffer and the appropriate restriction enzymes were added.

Fractionation of whole-cell extracts

All fractionation steps were performed at 4°C. A total of 200 mg of whole-cell extract prepared from WT BY4741 cells as described above were applied to a 20 ml Q-Sepharose Fast Flow column equilibrated with 20 mM Tris, pH 8.1. The column was washed with 60 ml of 20 mM Tris, pH 8.1 containing 2 mM EGTA, 2 mM MgSO₄, 2 µM pepstatin A, 0.6 µM leupeptin, 2 µg/ml chymostatin, 2 mM benzamidinium hydrochloride and 5 mM NaHSO₃. The column was subsequently washed with 60 ml of the above buffer containing 100 mM NaCl and 60 ml of buffer containing 500 mM NaCl.

The proteins in the 100 mM NaCl fraction that eluted from Q Sepharose (Q₁₀₀) were further fractionated on phenyl sepharose. Ammonium sulfate was added to the Q₁₀₀ eluate to a final concentration of 1.8 M. The proteins were applied to a 5 ml phenyl sepharose column

equilibrated with 50 mM Tris, pH 7.5 containing 1.8 M (NH₄)₂SO₄. The column was successively washed with three column volumes each of 1.8 M (NH₄)₂SO₄, 900 mM (NH₄)₂SO₄, 450 mM (NH₄)₂SO₄ and 225 mM (NH₄)₂SO₄ in 50 mM Tris, pH 7.5 containing 2 mM MgSO₄, 2 mM EGTA, 0.6 µM pepstatin A, 0.6 µM leupeptin and 2 µg/ml chymostatin. Following dialysis and concentration, the fractions were assayed for LLR activity.

Mass spectrometric analyses

To identify the proteins present in column fractions, samples were separated by SDS-polyacrylamide gel electrophoresis. The gels were fixed with 50% methanol and 10% acetic acid, and stained with SYPRO Ruby Red overnight. After rinsing with distilled water, proteins were visualized with the Kodak EDAS 290 system. Protein-containing bands were excised, placed in 20% methanol and submitted to the Nebraska Center for Proteomics (Lincoln, NE, USA) for mass spectroscopic analyses and protein identification.

Purified proteins and antisera

Pol δ, PCNA, RFC, RPA and FEN1 were overproduced and purified as described (32–35). The mutant enzyme Pol δ-01 was purified as described (36). Rabbit anti-mouse FEN1 antisera was a generous gift from Robert Bambara (University of Rochester).

RESULTS

Substrates used to monitor DNA large loop repair

Heteroduplex substrates were prepared with a loop on either the complementary (C) or the viral (V) strand (Figure 1). For example, C₂₇ denotes a loop of 27 nt located on the C strand. All substrates contain a site-specific nick on the C strand 114 bp 5' to the heterology. *In vivo* and *in vitro* studies in yeast have demonstrated the existence of two modes of correction of large loop heterologies: a loop-directed mode and a nick-directed mode (23,25). In loop-directed repair, the loop is removed irrespective of the presence of a nick. In nick-directed repair, the strand containing the nick is the target for excision and resynthesis. As shown in Figure 1A, the nick and loop are both on the complementary strand of C₂₇. Loop- and nick-directed repair therefore remove the loop to create the same EcoRI-sensitive product. In contrast, the nick and the loop are on opposite strands in the V₂₇ substrate (Figure 1B). Loop-directed repair is detected as EcoRI sensitivity due to removal of the loop. Nick-directed repair, which results in retention of the loop, yields sensitivity to NheI. We use the terms loop removal and loop retention to denote these two possible outcomes.

Yeast nuclear extracts and whole-cell lysates support LLR

Previous studies of LLR in *S. cerevisiae* utilized nuclear extract preparations (23,25,27). These studies showed that nuclear extracts efficiently repair unstructured loops of 16, 27, 30 and 216 nt. Although LLR activity is robust in

nuclear extracts, limited quantities of protein are obtained, which hindered efforts to fractionate extracts. Instead, we utilized a simpler and more rapid method of preparing large amounts of whole-cell extract (30). These whole-cell extracts are active for LLR, with repair rates 42–61% those in nuclear extracts on C₂₇ and V₂₇, including both loop removal and loop retention (Figure 2A). Whole cell lysates were further tested on a range of heteroduplexes, including a G/T mismatch and loops of 8, 16, 30, 45 and 216 nt (Figure 2B). Consistent with previously published data from nuclear extracts (23), repair of the G/T mismatch and C₈ loop removal occurred at low levels in whole-cell extracts. In contrast, loop removal was much more efficient on C substrates of 27–45 nt and V substrates of 16–216 nt (Figure 2B, unfilled bars). Whole-cell extracts also exhibited substantial loop retention activity for V heteroduplexes from 8 to 216 nt (Figure 2B, filled bars). Consistent with an earlier report (23), the differential repair activity on larger loops, compared to G/T and C₈ substrates, suggest that the repair reaction is specific for large loops rather than the result of nonspecific heteroduplex clipping by nucleases.

Identification of FEN1 as an LLR component

Neutralizing antibody experiments of yeast nuclear extracts identified Pol δ , RFC and PCNA as required components for LLR-dependent repair synthesis (27). To identify LLR protein(s) that act preceding repair synthesis, we used chromatography to identify activities that restore LLR in the presence of purified Pol δ , PCNA and RFC. Purified RPA was also included, although as shown later RPA is not essential for reconstitution. Chromatography of whole-cell extracts over Q Sepharose produced a fraction, eluted with 100 mM NaCl (Q₁₀₀), that was devoid of LLR activity on both C₂₇ and V₂₇ (Figure 3A). The addition of purified Pol δ , PCNA, RFC and RPA resulted in high levels of C₂₇ loop removal and V₂₇ loop retention activities (Figure 3A). V₂₇ loop removal was not observed in the Q₁₀₀ fraction, even with addition of purified proteins. These results indicate that nick-directed LLR, but not loop-directed LLR, occurs when the Q₁₀₀ fraction is added to Pol δ , PCNA, RFC and RPA (compare Figure 1 with Figure 3A). Since loop-directed LLR (i.e. V₂₇ loop removal) was not observed, these results also suggest that nick- and loop-directed LLR have different protein requirements. Similar results were obtained with fractionation of nuclear extracts (data not shown).

The Q₁₀₀ fraction from a whole-cell lysate was applied to a phenyl sepharose column. A fraction eluted with 450 mM (NH₄)₂SO₄ supported high levels of C₂₇ loop removal in the presence of Pol δ , RFC and PCNA (data not shown). The proteins from this column fraction were separated by SDS–PAGE, protein-containing bands were excised and analyzed by mass spectrometry. The structure-specific flap endonuclease FEN1 was among the proteins detected. An antibody against FEN1 recognized a ~43 kDa protein in the fraction from phenyl sepharose, confirming the mass spectrometry data (Figure 3B). Supplementation of PCNA, RFC and Pol δ with purified FEN1 reconstituted C₂₇ loop removal and

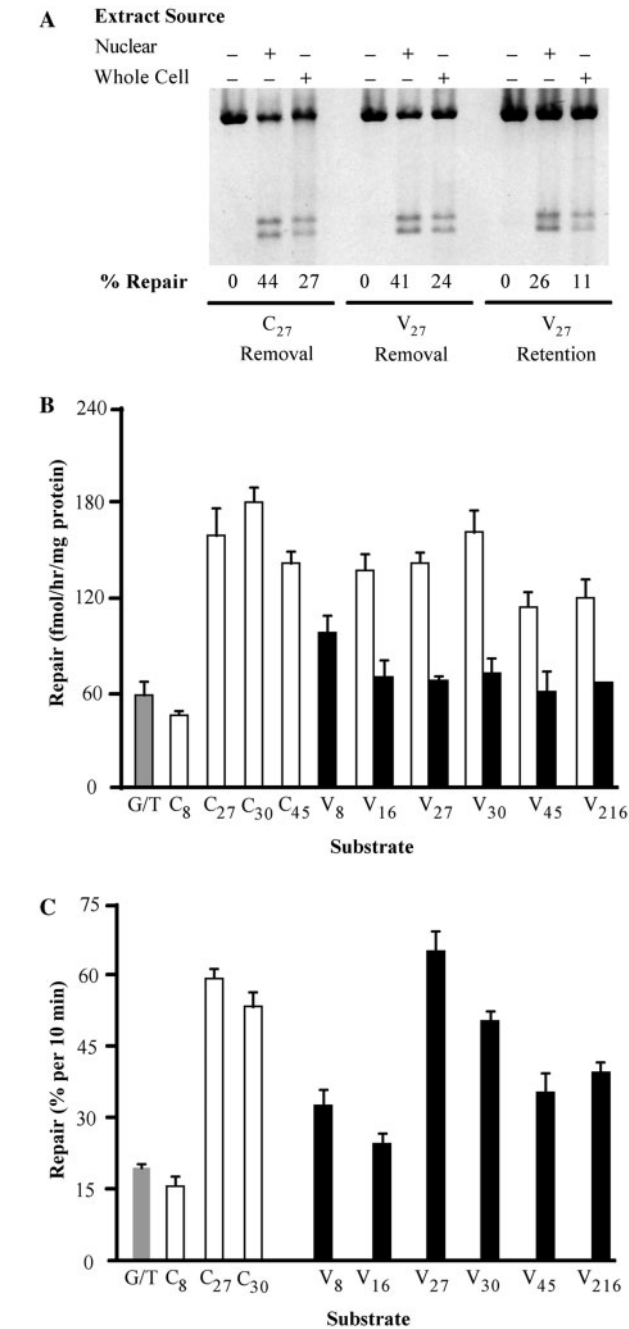


Figure 2. Yeast nuclear extracts and whole-cell lysates support large loop repair. Reactions scoring nick-directed repair (loop removal on C substrates; loop retention on V substrates) or loop-directed repair (loop removal for V substrates) were performed as described in Materials and methods section. (A) Nuclear extracts and whole-cell lysates were assayed for LLR activity on C₂₇ and V₂₇ in a one hour reaction (B) Whole-cell lysate repair activity was measured for the indicated heteroduplex substrates in a one hour reaction. Shaded bar, repair of G/T to A/T; unfilled bars, loop removal; filled bars, loop retention. Error bars indicate ± 1 SD. (C) Repair activity in a 10 min, 10 μ l reaction by 30 fmol FEN1, 30 fmol Pol δ , 60 fmol PCNA, 150 fmol RFC and 300 fmol RPA. Symbols are the same as for (B).

V₂₇ loop retention activities (Figure 3C). V₂₇ loop removal was not observed under these conditions (Figure 3C), nor at elevated levels of FEN1 (500 fmol) and PCNA (800 fmol; data not shown).

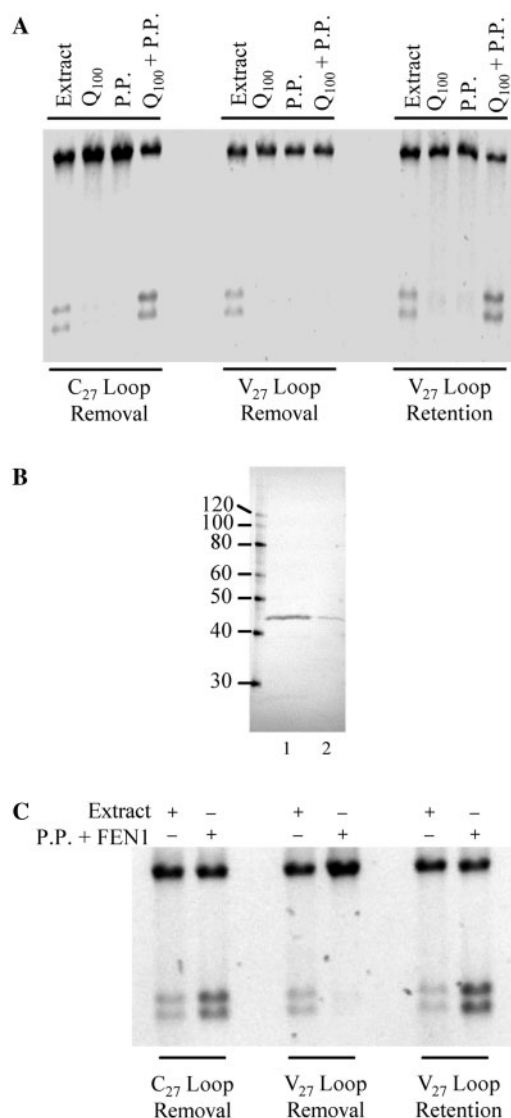


Figure 3. Identification of FEN1 as a LLR component. (A) Whole-cell extract was fractionated on Q sepharose, desalted and concentrated as described under Methods and materials section. LLR assays were performed for the starting extract, the Q₁₀₀ fraction (3 µg protein) and the Q₁₀₀ fraction supplemented with purified proteins ('P. P.') Pol δ (50 fmol), PCNA (300 fmol), RFC (50 fmol) and RPA (150 fmol). (B) Immunoblot analysis. The Q₁₀₀ fraction from whole cell lysate was applied to phenyl superose, and a fraction eluting at 450 mM ammonium sulfate was active for LLR when supplemented with the above purified proteins. Immunoblot analysis with anti-FEN1 antisera is shown for 20 µg of protein from the phenyl superose fraction (lane 1) or 2 ng of purified FEN1 (lane 2). Numbers on the left indicate molecular weight markers, with sizes given in kilo Daltons. (C) LLR assays of whole cell extract (1 h reaction) or 30 fmol Pol δ, 60 fmol PCNA, 150 fmol RFC, 300 fmol RPA and 30 fmol FEN1 (10 min reaction). For (A) and (C), the V₂₇ retention assay in extracts was increased to 1.5 h, to improve visibility of the repair products.

Specificity of nick-directed LLR in the partially reconstituted system

Several lines of evidence suggest that FEN1 is a relevant activity for nick-directed LLR. First, there is a similar pattern of LLR activity in the Q₁₀₀ fraction and with purified FEN1 (compare Figure 3A and 3C). Second,

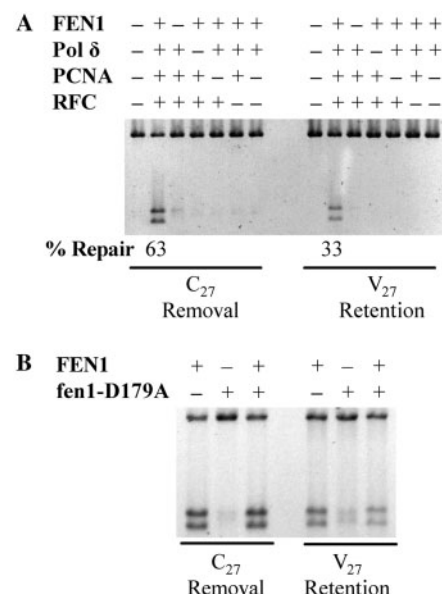


Figure 4. (A) FEN1, Pol δ, RFC and PCNA are necessary and sufficient for nick-directed repair of the C₂₇ and V₂₇ substrates. Each 10 µl reaction contained 30 fmol FEN1, 30 fmol Pol δ, 150 fmol RFC, and 60 fmol PCNA unless indicated otherwise. Reactions were initiated by the addition of 24 fmol C₂₇ or V₂₇ and were incubated at 30°C for 5 min. Aside from the specified values, the extent of repair was <5%. (B) Exonuclease-deficient FEN1, fen1-D179A, fails to support LLR in the reconstituted system. Reaction conditions were as in (A) except the incubation time was 10 min, 300 fmol RPA was added to V₂₇ reactions, and the presence of 30 fmol each FEN1 and fen1-D179A in the indicated lanes.

drop-out experiments showed that all four protein components (FEN1, Pol δ, PCNA and RFC) were required for C₂₇ loop removal and V₂₇ loop retention activities (Figure 4A). Omission of dNTPs from the reaction mixture also eliminated repair (data not shown). Third, the ratio of C₂₇ loop removal and V₂₇ loop retention was similar in the reconstituted system as in the whole-cell extract with about twice as much C₂₇ loop removal (Figures 2B and 4A). Fourth, assays were performed with the mutant enzyme fen1-D179A, which retains flap cleavage activity but is defective in exonuclease and gap endonuclease activities (37). This mutant enzyme failed to support LLR in the reconstituted system (Figure 4B), consistent with a model where FEN1 exonuclease activity is required for LLR (see Discussion section).

To further examine the relevance of FEN1 for nick-directed LLR, repair activity was measured on a number of substrates using the reconstituted system of FEN1, Pol δ, RFC and PCNA (Supplementary Table 2). The addition of RPA was also examined, because in other repair systems RPA has been shown to bind to and stabilize excision intermediates at single-stranded regions and removing secondary structure (38). RPA also stimulates the activity of FEN1 and facilitates repair synthesis by increasing the processivity of DNA polymerases (38,39). In our hands, addition of RPA had no discernible effect on low-level G/T repair, nor on loop removal on C substrates of 8, 27 or 30 nt loops. In contrast, RPA stimulated by about 2-fold V loop retention for loops of 8–216 nt

(Supplementary Table 2). Thus, the presence of RPA in the reconstituted system results in approximately equal levels of C₂₇ loop removal and V₂₇ loop retention. When the reconstituted LLR activity data with RPA were displayed graphically (Figure 2C), there was 3- to 4-fold higher LLR removal activity for C₂₇ and C₃₀ compared to repair of G/T or C₈. Similarly, V loop retention activity in the presence of RPA was 2- to 3-fold higher for most loops of 8–216 nt, compared to G/T. These results compare quite closely to the substrate specificity seen in whole-cell extracts (compare Figure 2B and C).

The RPA results provide additional support that FEN1 activity in the reconstituted LLR system is specific. Flaps up to 73 nt are efficiently cleaved by FEN1 (40). However, in the presence of RPA, flaps longer than about 25 nt are not a substrate for FEN1 because RPA coats the flap (40,41). We found that addition of RPA to our reconstituted system did not inhibit LLR; Supplementary Table 2 shows there was either no effect (for C substrates) or stimulation of repair (on V substrates). Since our ssDNA regions are long (e.g. 27 nt is long enough to bind RPA tightly) this is evidence that FEN1 acts in a coupled repair system, as opposed to nonspecific cleavage by FEN1.

While the results above support the idea of specificity in the reconstituted system, we addressed in one additional way the alternative possibility of a nonspecific, nick-translation-like reaction. In this scenario, Pol δ might displace 5' flaps that could be removed by FEN1. Processing in this manner from the 5' nick through the loop could potentially yield nonspecific repair. We tested this possibility with a mutant version of Pol δ , called Pol δ -01. Wild type Pol δ has an intrinsic 3' to 5' exonuclease activity that provides a proofreading function, and limits excessive strand displacement synthesis by the polymerase through a mechanism called 'idling' (42). Pol δ -01 is defective for this exonuclease activity and the mutant polymerase shows drastically increased strand displacement synthetic capacity. Therefore, one would expect that if flap displacement activity is important in our reconstituted LLR reaction, substituting Pol δ -01 for wild-type enzyme should enhance repair. Instead, we saw no evidence of accelerated LLR activity with Pol δ -01 (Figure 5). Time course reactions with wild-type Pol δ or with Pol δ -01 gave very similar results. Taken together, the specificity data and the Pol δ -01 result support the contention that the partially reconstituted system containing FEN1 conveys a substantial fraction of the specificity for large loops. It is of course possible that additional LLR factors, still unidentified, augment LLR specificity and/or repair efficiency.

The requirement for FEN1 exonuclease activity (Figure 4B) suggests that FEN1 and Pol δ cooperate to remove the 114 nt stretch between the nick and the loop. Presumably, this occurs by Pol δ -mediated DNA synthesis from the 3' end of the nick to create small flaps that are cleaved by FEN1 (42). If so, omitting dNTPs should limit synthesis and gap formation downstream of the nick. This idea was tested by examining restriction enzyme cleavage at sites close to the nick (DraIII) or close to the loop (BanII) in LLR reactions on C₂₇ with or without dNTPs. Figure 6A provides a schematic diagram of

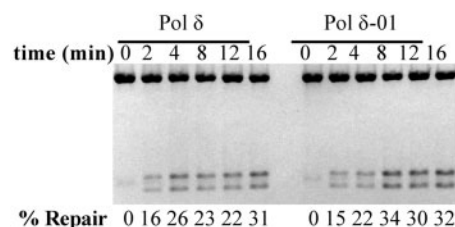


Figure 5. LLR assay using Pol δ or the exonuclease-deficient polymerase Pol δ -01. Each 10 μ l reaction contained 33 fmol C₂₇, 50 fmol FEN1, 50 fmol PCNA, 50 fmol RFC, 3.3 pmol RPA and 50 fmol Pol δ or Pol δ -01. The DNA was incubated with PCNA, RFC and RPA for 1 min at 30°C before the addition of FEN1 and Pol δ or Pol δ -01. Reactions were terminated at the indicated time periods and C₂₇ loop removal was scored as described in Materials and methods section.

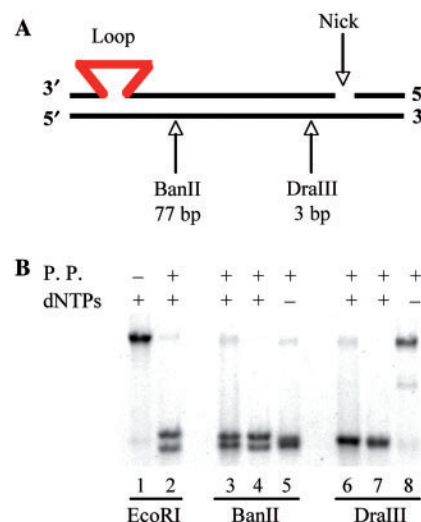


Figure 6. Analysis of single-strand gap formation in the absence of dNTPs. (A) Map of the diagnostic restriction enzyme sites relative to the nick and the loop on C₂₇. DraIII cuts 3 bp 3' of the nick, whereas BanII cleaves 77 bp 3' of the nick. Total distance from the nick to the loop is 114 bp. Digestion of C₂₇ with ClaI yields a 6.4 kb linear fragment (Figure 1A). Subsequent cleavage with either DraIII or BanII yields two smaller products of \sim 3.3 + 3.1 kb. (B) Each 10 μ l reaction, aside from lane 1, contained 60 fmol FEN1, 60 fmol Pol δ , 300 fmol RFC, and 120 fmol PCNA. The presence or absence of dNTPs is indicated. Reactions were initiated by the addition of 24 fmol C₂₇ and were incubated at 30°C for 15 min. After treatment at 70°C for 10 min to inactivate LLR, the DNA was treated for 1 h at 37°C with 6 U ClaI plus 5 U of either EcoRI (to monitor repair of the loop), BanII or DraIII.s.

the relevant sites, and Figure 6B shows the outcome. BanII cleavage occurred about equally well with or without dNTPs, suggesting the excision tract does not extend to this location 77 bp from the nick (compare lanes 3–4 with 5). There is some reduction in size of the upper band, consistent with partial excision, but clearly the BanII site remains intact because the DNA is almost completely cleaved to form the \sim 3.3 and 3.1 kb bands. In contrast, DraIII cleavage 3 bp from the nick was ablated by LLR in the absence of dNTPs, as shown by the presence of the 6.4 kb band (compare lanes 6–7 with 8). This result indicates that LLR-associated excision in the reconstituted system initiates at the nick, and that excision in the absence of dNTPs occurs over a limited range.

DISCUSSION

The correction of large looped heterologies in DNA is well documented, but the proteins that participate in LLR remained largely unidentified, until recently. Our previous work presented evidence with neutralizing antibodies and yeast extracts that Pol δ , RFC and PCNA are involved in the resynthesis step of LLR (27). The current study used fractionation and mass spectrometry to identify FEN1 as an additional LLR factor. Reconstitution experiments showed that purified FEN1, Pol δ , RFC and PCNA repair loops of 8–216 nt in a 5' nick-directed manner, regardless of which strand contained the loop. RPA was stimulatory on some substrates, but not required for repair. In contrast to nuclear and whole-cell extracts, however, nick-independent loop removal was not detected with the purified proteins. This finding indicates that nick-stimulated and loop-stimulated LLR require a different set of proteins, some of which remain unidentified. Nonetheless, the reconstituted system showed a similar substrate specificity profile as seen in whole-cell extracts for nick-directed repair. Furthermore an exonuclease-deficient Pol δ variant protein, Pol δ -01, with increased flap-displacement activity was no different than wild-type Pol δ in the reconstituted reaction. The substrate specificity data and the Pol δ -01 argue against a simple nick-translation type model. Instead, the results suggest partial reconstitution of 5' nick-directed LLR with FEN1, Pol δ , RFC and PCNA. While additional factors may enhance the activity or specificity of the reaction, to our knowledge this is the first report of a biochemically defined system for LLR.

FEN1 is the first nuclease to be implicated in mitotic LLR. This finding adds to the impressive list of activities in which FEN1 participates, including Okazaki fragment processing, long patch base excision repair and nonhomologous end joining (43–45). Other studies showed that FEN1 interacts functionally and physically with Pol δ , PCNA and RPA (43–45), lending additional support to the idea that these four proteins plus RFC could catalyze 5' nick-directed LLR.

How might this group of five proteins act to elicit repair of large loops? Excision tract mapping of 5' nick-directed LLR in nuclear extracts demonstrated that repair involves excision of the DNA from the nick to a location past the loop (12,13,19,23). Based on the data in Figures 4B and 6, and the existing literature, 5'-nick directed repair by purified FEN1, Pol δ , PCNA and RFC likely proceeds by initiation at the nick. Initial cleavage at the loop is not supported by our data; otherwise, V27 loop removal would be expected but it was not observed (Figure 3C). Furthermore, FEN1 in general does not efficiently cleave loops or bubbles (46,47), and the putative gap endonuclease activity reported for FEN1 requires significantly higher enzyme concentrations than used in this study (48,49). In our current working model, recognition of the free 3'-OH at the nick by RFC results in the loading of PCNA and subsequent recruitment of FEN1 and Pol δ to the nick. FEN1 cleavage near the nick would primarily produce a 1-nt gap; it is unlikely that FEN1 alone is responsible for excising the DNA all the way from the

nick to the loop since its exonuclease activity is relatively weak. It is more likely that excision requires the concerted activity of Pol δ and FEN1 in a manner similar to that observed in Okazaki fragment processing, where flaps are formed by displacement synthesis and then simultaneously cleaved (42). Thus, one putative function of FEN1 in LLR is to assist in traversing between the nick and the loop. Once the PCNA/Pol δ /FEN1 complex reaches the 5' side of a loop on the C strand, a flap will be generated that is equal in length to the original loop. FEN1 could then track along the flap and endonucleolytically cleave it at the base. By this model, FEN1 endonuclease activity at the 5' side of the flap is also required for LLR. For a loop on the V strand, an intact single-stranded region rather than a flap would occur. RPA may stimulate repair synthesis through the single-stranded region. Final processing of both C and V containing loops would require sealing of the nick by DNA ligase, although we did not test this prediction directly. A likely candidate for nick-directed LLR is DNA ligase I since it interacts with and is stimulated by PCNA. Cdc9, the *S. cerevisiae* homolog of DNA ligase I, was detected in the PS₄₅₀ fraction by mass spectroscopy (Sommer, D. and Lahue, R. unpublished observations). Ligase I has been shown to limit the length of nick translation patch lengths and nick ligation is the signal for the unloading of PCNA by RFC (41).

Kolodner and colleagues (50) originally showed that *rad27* mutant strains, lacking FEN1, accumulate duplication mutations of 5–108 bp flanked by direct repeats of 3–12 bp. This phenotype was attributed to the production of displaced 5' flaps in the absence of FEN1, followed by annealing of the 5' end of the flap to a region of homology. The flaps could be ligated to create a covalent looped heteroduplex, which would template for a duplication mutation in a subsequent round of replication. Thus, the belief was that FEN1 acted only to cleave 5' flaps and therefore prevent duplications. Our results with LLR reconstitution lead to the hypothesis that *rad27* mutants not only produce excess flaps, but these mutants may also be at least partially defective in their repair of the looped heteroduplex by LLR. Thus, the duplication phenotype associated with *rad27* mutants may be due to both excessive flap production and defective loop repair. In support of this idea, the size range of mutations observed in *rad27* strains, 5–108 bp (50), overlaps with the range of large loops repaired in the partially reconstituted LLR reaction described here. However, a yeast *Arad27* strain can repair large loops *in vivo* (25) and extract from a *Arad27* mutant was also LLR proficient *in vitro* (this study; data not shown). Therefore, there may be one or more nucleases that are functionally redundant to FEN1 that could replace it in 5'-directed LLR. Precedent for partial redundancy with FEN1 comes from studies of Okazaki fragment maturation, where backup activities of Exo1 and Dna2 are likely involved (45). Mismatch repair in *E. coli* also requires several redundant nucleases (51). Accordingly, we found that wild type yeast extract produced at least one additional active fraction from phenyl sepharose, eluting at a different salt concentration than FEN1. This additional fraction contained Rad2, as shown by mass spectrometric analysis, and purified

Rad2 added to Pol δ , PCNA and RFC gave LLR activity, albeit at a significantly reduced level compared to the FEN1-containing system. The potential role of Rad2 in LLR remains to be elucidated.

In summary, fractionation of yeast lysates revealed that 5' nick-directed repair of large loops can be separated from loop-directed repair. Further fractionation lead to the identification of FEN1, a nuclease that in combination with Pol δ , PCNA, and RFC is able to support 5' nick-directed repair of 27 nt loops. These factors may assemble at the nick and act in a concerted fashion to excise and re-synthesize the DNA, thus correcting the heterology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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